A METHOD FOR MAKING CARBOHYDRATE SERUM BROTH OF CONSTANT COMPOSITION FOR USE IN THE STUDY OF STREPTOCOCCI *

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For several years I have been studying the cultural and biological characters of the streptococcus group of the coccaceae, and have given particular attention to the development of the most favorable medium.

I learned very early in the work that in order to obtain good growth of the streptococci, fluid media were preferable to solid media. I also soon learned that the addition of serum to the media enhanced very markedly the development of the streptococci. It was often observed that exudate from appendiceal and other peritonitis cases showed in direct smears a mixture of Gram-negative bacilli and Gram-positive streptococci. Part of this material was added to plain broth and part to serum broth, and on examination of the growth in twenty-four hours or less the microscopical pictures from the two media were very different. That from the plain broth showed a predominance of Gram-negative bacilli with very few Gram-positive cocci, while the smear from serum broth showed what often appeared to be a pure culture of streptococci. From feces, one obtains practically the same results, that is, in the serum broth, the streptococci developing are numerous, while in plain broth they are almost absent.

That the addition of serum increases the growth of streptococci is, of course, well known. Elschnig[1] employed it in making cultures from the conjunctiva, and the high percentage (30) of streptococci which he obtained from apparently normal eyes is undoubtedly due to the use of the serum medium.

In studies on the pneumococcus the use of serum media was early recognized as necessary and was widely employed.

Hiss[2] used serum in his serum water media which were made as follows: Beef serum one part, distilled water two or three parts, heated in the Arnold sterilizer for 12 minutes at 100 C. One per cent. of a 5 per cent. aqueous solution of litmus was added until a deep transparent blue was obtained. One per cent. of the sugar to be used in the test was added to this medium. The serum water media were sterilized by the fractional method of 100 C.

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1. *Deutsch. med. Wchnschr.*, 1910, 26, p. 1229.
2. Hiss and Zinsser, Text-Book of Bacteriology.

Buerger,[3] modifying these media by adding about 2 per cent. peptone, obtained better results in growing the pneumococcus than with Hiss' serum waters.

Ruediger[4] further modified the serum water media. He employed beef serum which was diluted with an equal volume of water, passed through a large Berkefeld filter, heated to 65 C. for one-half hour on two successive days, and added in equal amounts to the previously tubed and sterilized litmus carbohydrate peptone and salt solution.

Buerger,[5] in an article on the differentiation of streptococci, points out that the addition of ascitic fluid not only enhances growth but seems to favor the fermentation of certain of the carbohydrates that were not attacked in the simple media (sugar free broth), and lays emphasis on the use of the most favorable media for the growth of these organisms in doing the fermentation tests.

Heinemann[6] used serum broth for "rejuvenating" the streptococcus lacticus before injecting it into rabbits. Marmorek used various strengths of different sera for producing rich and virulent cultures of streptococci, recommending equal parts of peptone broth and human serum as the best.

In an attempt to differentiate the large numbers of streptococci that were obtained in this laboratory carbohydrates were used as recommended by Gordon.[7] The media used differed, however, in that the broth was made from Liebig's meat extract and serum added in the proportion of one part serum to four of the broth. The serum employed was usually hydrocele fluid but ascitic and ovarian fluids were also used. The two latter were very often found to be of little use in enriching our media. All three sera were moreover quite inconstant in their albuminous content and the results obtained were, at times, quite irregular. This led to the attempt to make a serum broth on which one could rely for always obtaining a good growth of streptococci. After we had tried several of the serum waters and found them, although a decided improvement on plain broth, not as satisfactory as the serum broth, the following serum medium was prepared which was later improved.

Beef blood was collected from the abattoir in sterile quart jars, such as are commonly used for storing preserves, called lightning jars. The blood was allowed to clot in the cool room of the abattoir for about 15 minutes. It was then brought to the laboratory, rimmed, and allowed to stand overnight in a cool place. The clear serum was obtained by centrifugalization, after which it was ready for use. One hundred c.c. of this serum was added to 300 c.c. of distilled water, and sterilized for 15 to 20 minutes in flowing steam on three successive days. The mixture became slightly milky or opalescent but was perfectly transparent.

3. *Jour. Exper. Med.*, 1905, 7, p. 524.
4. *Jour. Infect. Dis.*, 1906, 3, p. 756.
5. *Jour. Exper. Med.*, 1907, 9, p. 428.
6. *Jour. Infect. Dis.*, 1907, 9, p. 87.
7. *Ann. Rept. Loc. Gov. Bd., London*, 1903-4, p. 388.

The carbohydrate broth was made as follows:

Peptone (Witte)	40 gm.
Meat extract (Liebig's)	12 "
Sodium chlorid	20 "
Distilled water	1,000 c.c.

This broth was four times the usual strength. It was made neutral to phenolphthalein (hot titration) and 4 gm. of the carbohydrate and 4 c.c. of Andrade's indicator were added to 100 c.c. This broth was sterilized in flowing stream for 15 to 20 minutes on three successive days.

The sterile serum water was then mixed with this quadruple strength carbohydrate broth, and the medium, consisting of 1 part serum to 4 parts 1 per cent. carbohydrate broth, was tubed into sterile tubes by means of a sterile tubing-funnel, or by use of a sterile syphon. The use of the syphon was as follows: The 500 c.c. of carbohydrate serum broth was mixed in a large flask. A rubber cork was fitted with a syphon and a glass tube bent at right angles, the outer end of which was plugged with cotton wool. This apparatus was sterilized in another flask, transferred to the flask containing the medium, the syphon started by blowing through the bent tube, and the medium tubed into sterile tubes.

This medium gives a much better growth of streptococci than the serum waters or ordinary broth. It is coagulated on the production of acid in the fermentation of the carbohydrates as is the case in all heated serum media (Longcope).

The heating of the serum undoubtedly alters many of its albuminous constituents, possibly they are changed into the so-called colloidal state as Longcope[8] suggests. Whatever the change, it is not as favorable a medium for streptococcus growth as the unheated serum.

In order, therefore, to obtain a medium containing unheated serum it was decided to sterilize the serum by filtration. It was found, however, that the filter became clogged after a relatively small amount of the serum had passed through. To overcome this, the serum was diluted one-half with distilled water and it now passed vithout any difficulty through an ordinary Berkefeld filter. To assure sterility, it is important that the filtration takes place slowly as otherwise organisms pass through the filter candle.

A very useful addition to the ordinary filtering apparatus is the insertion of a large glass tube into the rubber cork of the filtering flask to direct the filtered serum past its side opening. Also a large inverted test tube covering the filter candle makes possible the filtration of the last of the serum without the bubbles that are commonly formed.

The carbohydrate broth with which the serum was subsequently mixed was made up as follows:

Double strength broth + 1.2 ac	200 c.c.
Distilled water	100 c.c.
Carbohydrate	4 gm.
Andrade's Indicator	4 c.c.

Sterilized in flowing steam 15 to 20 minutes on three successive days, cooled and then 200 c.c. of the diluted and filtered serum added.

The finished medium gave a carbohydrate serum broth. It was tubed as above described and was incubated for several days before use to ensure sterility.

8. *Jour. Exper. Med.*, 1905, 7, p. 131.

Different strengths of serum are easily obtained by varying the above formulae. The dilute serum obtained as described above is very useful to add to carbohydrate agar for anaerobic cultures.

The advantages of this method for making serum broth are: (1) A uniform mixture is obtained in all tubes; (2) there is less liability for contamination than by the use of sterile pipets and the addition of the serum to each tube; (3) the serum used has never been heated and is, therefore, unaltered; (4) the use of beef serum assures a serum of reasonably constant composition and it is, therefore, useful for comparative tests. Moreover, as Longcope[9] has pointed out, beef serum does not show the production of acid with the growth of pneumococcus and in my experience acid is never produced in the control sugar-free serum broth with a great variety of streptococci; (5) this serum medium is not coagulated by the production of acid, only a slight opalescence appearing when much acid is produced.

I have carried out a number of experiments with the streptococcus by growing it in ordinary carbohydrate broth and in the carbohydrate serum broth, and our results have been striking. I have found that many strains of streptococci fail to grow at all while others grow very poorly in ordinary broth. It is true, on the other hand, that a great number grow well in both media, but always more luxuriantly in the serum broth. It is, I believe, fundamental in the study of the fermentation reactions of the streptococci, to have a medium in which the organisms grow well independently of the carbohydrate added. Although many of the streptococci will ferment a certain carbohydrate in broth, serum water, or serum broth, others grow so poorly in the former that they fail to attack the carbohydrate, and the result would appear negative if no further study were undertaken.

In a recent article Broadhurst[10] has shown that she gets a higher acidity in meat than in meat extract broth. It is well known that meat infusion broth makes a better medium for sensitive organisms than meat extract broth, and I would be inclined to believe that she was dealing with a more vigorous growth in the former case.

Floyd and Wolbach[11] on the differentiation of streptococci say, "We are not certain that the clotting of milk is solely dependent upon carbohydrate fermentation. This is tentatively offered in the light of our experience in that milk may be acidified without acid production in dextrose and lactose serum waters." It is interesting to note in this connection that out of 63 strains in their Group 2, 29

9. Ibid.
10. *Jour. Infect. Dis.*, 1913, 13, p. 404.
11. *Jour. Med. Research*, 1914, 29, p. 493.

show acid in milk and 34 clotting of milk without producing any acid in lactose. In Group 4, they found that out of 42, 8 produced acid and 3 clot in milk, without acid in lactose serum water. In Group 5, out of 43, only 1 produced clot in the milk without acid in the lactose medium. In Group 6, the results in milk and in lactose serum water are the same with the exception that two strains failed to affect either the milk or the lactose serum water.

Group 2 "corresponds closely to streptococcus pyogenes." Group 4 is composed of non-hemolytic strains and is "intermediate between streptococus pyogenes and the streptococcus anginosus." Group 5 "corresponds closely to streptococcus anginosus."

The more strictly parasitic streptococci are grown with greater difficulty in artificial media and I would suggest for these confusing results the explanation that the growth in the lactose serum water is not vigorous enough to allow the organisms to exert their fermentative powers, while in the milk the conditions for growth are more favorable and the organisms ferment the contained lactose and dextrose. The explanation of the results of Group 1, where no fermentative action was demonstrated or only acid was developed in dextrose, is more difficult as milk is not affected. "These cultures come mostly from cases where the streptococcus played a pathogenic role." I have met in investigations of about 500 strains a fair number (25) of streptococci which failed to ferment lactose, but which hemolysed blood. They are similar to the 44 out of 62 strains observed by Floyd and Wolbach. I have also encountered a few strains (12) which do not hemolyse and which correspond to the streptococcus equinus of Andrewes and Horder.[12] However, I have always noted a slight acidity in litmus milk and acid was formed in the dextrose and saccharose serum broth. The five strains in Group 3 and one in Group 5, as reported by Floyd and Wolbach,[13] which appear to ferment the lactose in the serum water media but fail to affect any change in milk, are interesting and would bear further investigation.

Winslow and Palmer,[14] in a comparative study of intestinal streptococci, "utterly failed" to isolate streptococci from feces by growing in dextrose broth preliminary to plating on agar and resorted to plating on agar directly, which method "proved generally successful."

From my experience, I feel certain that the use of serum broth would have materially helped them in their isolations. These workers also had in their carbohydrate broth fermentation tests a considerable number of "clear tubes" in which no obvious growth of the streptococci had occurred. These tubes were plated out on agar with the result that out of 49 tubes plated "3 showed many colonies, 15 showed 1 to 6 colonies, and 31 showed none." "It may reasonably be assumed," they say, "that in such cases the streptococci introduced had simply failed to develop and gradually died out on account of the lack of suitable carbohydrate pabulum, on which these organisms appear to be highly dependent." It would seem that these streptococci which failed to develop and others in which feeble growths had occurred had not been given the most favorable environment in which they could demonstrate their fermentative powers.

Salomon[15] and other German workers in studying the fermentative powers of the streptococci have used litmus ascites carbohydrate agar. Their results are very different from those obtained by others where fluid media were employed. Salomon found that 13 strains of the pneumococcus and 6 out of 10 of his strains

12. *Lancet*, 1906, 2, p. 708.
13. *Jour. Med. Research*, 1914, 29, p. 493.
14. Systematic Relationships of the Coccaceae.
15. *Centralbl. f. Bakteriol.*, Abt. I, Orig., 1908, 47, p. 1.

of streptococcus mucosus practically failed to show any fermentative powers by his method, although he used 18 different carbohydrates. These organisms are well known to have high powers of fermentation, and the results Salomon obtained are due, we believe, to the use of solid in preference to fluid media.

Winslow[16] in his study of the coccaceae draws attention to the fact that in obtaining material by the method of plating on agar and incubating at 20 C. he failed to obtain many of the more strictly parasitic streptococci which grow only feebly on solid media and are most active at a temperature of 37 C. In this I agree perfectly, but would go further and say that I believe a study of streptococci without the use of serum broth loses much of its value, from failure to isolate many of the less easily grown strains, and furthermore, many are not given a sufficiently rich medium in which they may develop vigorously enough to exercise their fermentative action on the contained carbohydrate.

There is a well recognized difference in cells in respect to their ability to exercise their specific functions. These functions may be entirely absent where the cells are merely living or even slowly reproducing. A healthy state of the cell where both reproducing and functional activities are at their highest is to be found only where the environment is best suited to the needs of the cell.

This is well known when we consider the functional activity of the cells in the metazoa and is, I believe, equally true among the unicellular organisms now under discussion.

CONCLUSIONS

Serum broth is the most favorable medium for the isolation and growth of streptococci. It is so well suited to the growth of streptococci that in mixed cultures, even vigorous organisms such as the bacillus coli are overgrown in twenty-four hours.

Cultures containing different forms of streptococci should be planted in serum broth for at least twenty-four hours before plating on blood agar. If this is not done, many of the more pathogenic forms are liable to be overlooked

Many strains of streptococci grow poorly in plain carbohydrate broth while others fail to show any growth at all.

In testing the fermentative powers of streptococci the carbohydrates should be added to serum broth.

The method here described offers a means for making a carbohydrate serum broth of reasonably constant composition, and which, in my hands, has always given a good growth of streptococci.

16. Systematic Relationships of the Coccaceae.